Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Identification of Vaccine Strains of Newcastle Disease Virus

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1. Introduction

This Supplemental Assay Method (SAM) describes *in vitro* methods to identify the domestic vaccine strains of Newcastle disease virus. The method uses plaque formation in differential media, plaque morphology, and the elution rate from hemagglutinated chicken erythrocytes. Elution or nonelution is confirmed by hemagglutination upon resuspension of the chicken erythrocytes.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Centrifuge (Beckman J-6B, JS-4.2 rotor)

2.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model No. 252)

2.1.3 Water-jacketed incubator with a humidified 5% CO_2 atmosphere and temperature set at 37°C, (Forma Scientific, Model No. 3158)

2.1.4 Vortex mixer (Thermolyne Maxi Mix II, Model No. M37615)

2.1.5 Magnetic stir plate

2.1.6 Scissors, sterile (Roboz RS-6800)

2.1.7 Curved tip forceps, sterile (V. Mueller SU 2315)

2.1.8 Microliter pipette (Rainin Pipetman, P1000)

2.1.9 250-ml trypsinizing flask with stir bar, sterile

2.1.10 Erlenmeyer flask with a stirring bar, sterile

2.1.11 Hemocytometer

2.1.12 Bunsen burner

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Cotton swabs

2.2.2 Tissue culture dish, 150 x 10 mm (Falcon, Cat. No. 1058)

2.2.3 Tissue culture dish, 100 x 20 mm (Falcon, Cat. No. 3003)

2.2.4 Plastic funnel covered with 4 layers of fine gauze

2.2.5 Polypropylene conical tube, 29 x 114 mm, sterile, 50 ml (Sarstedt, Cat. No. 62.547.205)

2.2.6 Polypropylene centrifuge tubes, 250 ml (Corning, Cat. No. 25350)

2.2.7 Roller bottles, 1000 ml (Falcon, Cat. No. 3007)

2.2.8 Serological pipettes (Falcon, Cat. No. 7530)

2.2.9 60-mm culture dish, tissue culture treated (Costar, Cat. No. 3160)

2.2.10 2 dozen specific-pathogen-free (SPF) chick embryos, 9- to 11-day-old

2.2.11 Fetal bovine serum (FBS)

2.2.12 L-Glutamine (Sigma, Cat. No. G7513)

2.2.13 Trypsin, 0.25% (Cello Corporation, Cat. No. AT25)

2.2.14 Pipette tips (Rainin 0-100, 0-200, 100-1000)

2.2.15 Ionagar No. 2S

2.2.16 Solutions

All solutions are autoclaved or filter sterilized.

1. Dulbecco's phosphate-buffered saline (PBS)

Solution A (Ca++- and Mg++-free PBS)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Phenol Red	0.04 g
q.s. with distilled or deionized water	1000 ml

Solution B

$CaCl_2$	1 g
q.s. with distilled or deionized water	100 ml

Solution C

$MgCl_2 \cdot 6H_2O$	0.1 g
q.s. with distilled or deionized water	100 ml

Autoclave the 3 solutions separately and cool before mixing. Mix 800 ml of solution A and 100 ml each of Solution B and C for a total volume of 1000 ml. This is a 1X solution. Use solutions A + B + C as a diluent for virus suspensions and solution A as washing saline for cell cultures.

2. Growth Medium for CEF Primaries

Earle's BSS with 0.65% lactalbumin hydrolysate: without sodium bicarbonate	90%
Minimum essential medium, vitamins (100 X)	1%
Minimum essential medium, amino acids (50 X)	2%
Fetal bovine serum, heat inactivated	5%
Penicillin, 10,000 units/ml	1%
Streptomycin, 10,000 ug/ml	1%

Add 7.5% sodium bicarbonate solution to adjust pH to 7.2 to 7.4.

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3. Additive Solutions

Solution D

DEAE-dextran	1 g
q.s. with distilled or deionized water	100 ml

Autoclave at 120°C for 15 min.

Solution M

$MgSO_4 \cdot 7H_20$	49.2 g
q.s. with distilled or deionized water	100 ml

Autoclave at 120°C for 15 min.

4. First Overlay Medium (2X)

2 X Earle's BSS with 1.30% lactalbumin hydrolysate: without sodium bicarbonate	80%
Minimum essential medium, vitamins (100 X)	2%
Minimum essential medium, amino acids (50 X)	4%
Fetal bovine serum, heat inactivated	10%
Penicillin, 10,000 units/ml	2%
Streptomycin, 10,000 ug/ml	2%

Add 7.5% sodium bicarbonate solution to adjust pH to 7.2 to 7.4.

5. Mix this 2X medium with equal parts of:

Ionagar No. 2S	1.6%

Autoclave at 120°C for 15 min.
Add to Ionagar after autoclaving:

DEAE-dextran, 1% solution (Solution D)	4.0%
$MgSO_4 \cdot 7H_2O$, 2 M solution (Solution M)	3.0%

Mix cooled agar and medium well and bring to 44° to 45°C in a water bath.

6. Second Overlay Medium

The second overlay medium is the same as the first with the addition of neutral red.
Neutral red, 1% solution (1% of total agar medium)
Store at 4°C.

7. Differential Medium without DEAE-dextran and Magnesium Sulfate

First and second overlays are prepared as above except DEAE-dextran and magnesium sulfate solutions are excluded.

8. Alsever's Solution

Glucose	20.5	g
Trisodium citrate dehydrate	8.0	g
Citric acid monohydrate	0.55	g
Sodium Chloride	4.2	g
q.s. with distilled or deionized water	1000	ml

pH 6.1

Sterilize by membrane filtration through a .45-µm filter.

9. 0.01 M PBS

Disodium phosphate anhydrous	1.096	g
Monosodium phosphate monohydrate	0.315	g
Sodium chloride	8.5	g
q.s. with distilled or deionized water	1000	ml

pH 7.2
May be stored at 4°C for 3 to 4 wk

2.2.17 Cell cultures

Primary chicken embryo fibroblasts (CEF) are used for this procedure. Chicken embryos are prepared by removing all viscera, heads, and appendages, then trypsinized with BPL-treated trypsin, rinsed with CA++- and Mg++-free PBS and seeded into 60 x 15-mm plastic petri plates at the rate of 10^7 cells in 5 ml of growth medium per plate. A more rapid method of plating cells without counting is by suspending 1 ml of packed cells (200 X *g* for 10 min) in 100 to 150 ml of growth medium and plating 5 ml of this suspension. Confluent monolayers are formed in 18 to 24 hr.

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3. Preparation for the test

3.1 Personnel qualifications/training

The executor must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals. The executor must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the Center for Veterinary Biologics-Laboratory (CVB-L) or equivalent; and training in the operation of the necessary laboratory equipment listed in part **2.1**.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturer’s instructions and monitor in compliance with current corresponding CVB-L/National Veterinary Services Laboratories standard operating procedures (SOPs) or equivalent.

3.3 Preparation of reagent/control procedures

Prepare reference viruses in the same manner as sample preparation.

3.4 Preparation of the sample

3.4.1 Rehydration of vaccine

The vaccine is reconstituted with accompanying diluent as directed by the manufacturer. This becomes the log 10^0 dilution. In the case of a desiccated poultry vaccine to be used with a large volume of water, rehydrate the vaccine with 30 ml of sterile purified water per 1000 doses.

3.4.2 Dilutions of vaccine

Serial tenfold dilutions are made from the reconstituted vaccine by diluting 0.5-ml amounts of vaccine into test tubes containing 4.5 ml of Dulbecco's PBS as diluent.

4. Performance of the test

4.1 Method for assaying for plaque formation

4.1.1 Inoculation of cell cultures

Gently wash each monolayer with 3 ml of warm (35° to 37°C) Dulbecco's PBS before inoculation. Drain and aspirate each plate. Inoculate 0.1 ml from log 10^{-3} through log 10^{-5} dilutions into each of 6 plates. The plates are rocked to distribute virus inoculum.

4.1.2 Absorption of virus

Inoculated plates are incubated at 37.5°C $\pm$ 0.5° for 60 min in a humidified CO_2 (3% to 5%) incubator. Maintain high humidity during this period to prevent drying of the monolayer. It is not necessary to wash or drain the monolayers when 0.1 ml of sterile inoculum is used.

4.1.3 First agar-medium overlay

1. Three plates of each dilution are overlayed with agar-medium containing Solution D and Solution M. Three plates of each dilution are overlayed with agar-medium without these additives.

2. Specifically, divide agar-medium into equal parts and add Solution D and Solution M to 1 part but omit them from the other part. With the agar-medium at a temperature of 44° to 45°C, gently add overlays to the plates. After the agar-medium overlay has solidified, the tray of plates is carefully returned to the CO_2 incubator. Exercise care in handling the tray in order not to jar the plates.

3. Incubate plates at 37.5°C $\pm$ 0.5° for 72 hr.

4.1.4 Second agar-medium overlay

The second agar-medium overlay is applied 72 hr after the first. Then, the monolayer is allowed to absorb the neutral red stain. A minimum of 4 hr is necessary for color contrast to form in the monolayer.

4.1.5 Reading plaque morphology

The broad meaning of "plaque" is that it is a patch on a surface. In this work, plaque refers to a necrotic patch in a cellular monolayer. Viable cells stain red, while the necrotic areas caused by viral infections do not stain.

Plaques are most easily distinguished at 96 hr after inoculation. Characteristics of plaque formation under each agar-medium preparation are recorded. Plaque size in mm, turbidity as turbid or clear, and morphology as shape and evenness of edges are recorded. Under agar-medium containing DEAE-dextran and $MgSO_4$, the B1 strain (NDV-B1) and the LaSota strain (NDV-LaS) produce pinpoint to 1.5-mm plaques of varying turbidity. The titer of the virus may be calculated by counting the plaques. Under agar-medium without DEAE-dextran and $MgSO_4$, NDV-B1 and NDV-LaS usually do not produce plaques. Occasionally, cytopathic effects are observed that later form plaques, but this is not reproducible.

4.1.6 Reference Control Virus

NDV-B1 and NDV-LaS viruses are included in this test for reference observations. Log 10^{-3} and log 10^{-4} dilutions are inoculated into 2 plates per each dilution, following the same procedure as described above. Optimum concentration is 30 to 150 plaques per plate, and only counts within this range are considered for quantitation of virus.

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4.2 Method for assaying hemagglutination-elution (HA-E) and hemagglutination-resuspension (HA-R)

4.2.1 The method of production of most NDV vaccines eliminates the hemagglutinin commonly found therein. This necessitates an additional step in characterizing the vaccine strain. Inoculate 0.1 ml from the log 10^{-3} dilution into the allantoic cavity of 5, 9- to 11-day-old chicken embryos. Harvest amnio allantoic fluids 3 to 4 days later. This is used in the HA-E and HA-R tests.

4.2.2 Collection of Chicken Erythrocytes (C-RBC)

The erythrocytes are collected at a 1:4 dilution in Alsever's solution. They are washed 3 times and stored in Alsever's solution. Erythrocytes, not over 48 hr old, are freshly prepared at each time of use.

4.2.3 Preparation of C-RBC

0.5 ml of washed and packed C-RBC are resuspended in 99.5 ml of 0.01 M PBS.

4.2.4 Classical Tube Test

Serial twofold dilutions of each virus tested, from 1:5 to 1:10,240, are made in 12 x 75-mm test tubes containing 0.5 ml-vol of 0.01 M PBS. Diluent and erythrocyte controls are included. One-half (0.5) ml of the erythrocyte suspension is added, and the test is set at 4°C until observations of the hemagglutination and elution patterns are made. Hemagglutination patterns are read at 1 and 2 hr, continuing every 2 hr (during regular working hours) for a total of 24 hr. Results are recorded. At the end of 24 hr, the test rack is shaken to uniformly resuspend the erythrocytes. Two hr after resuspension, the hemagglutination is again read and recorded. NDV-B1 and NDV-LaS reference viruses are included as positive controls in the HA-E and HA-R tests.

5. Interpretation of the test results

5.1 The difference between rapid and slow elution is based upon at least 2 observations: (1) the elution pattern, and (2) the hemagglutination pattern after resuspension. Complete elution that occurs within 24 hr is contrasted with failure to do so. NDV-B1 will elute within 2 hr. NDV-LaS remains hemagglutinated after 24 hr even though some elution may occur.

5.2 Confirmation of elution is made by the HA-R test. Failure to hemagglutinate after resuspension at 24 hr is contrasted with hemagglutination. NDV-B1 will not hemagglutinate on resuspension of erythrocytes, while NDV-LaS will hemagglutinate and often the titer will increase.

Summary of Identification of Vaccine Strains of Newcastle Disease Virus:

Plaques Formed in Differential Media		Hemagglutination	
With DEAE-dextran and $MgSO_4$	Without DEAE-Dextran and $MgSO_4$	Elution Time	Resuspension
NDV-B_1 +	-	$\leq$24 hr	-
NDV-LaS +	-	$\geq$24 hr	+

6. Report of test results

Sample is reported out as NDV vaccine strain B_1 or LaSota based on results of identity testing.

7. Changes

This document was rewritten to meet the current CVB-L QA SAM format. No significant changes were made from the previous protocol. This document supersedes the March 1, 1986, version.